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Role of Protein Phosphorylation in the Regulation of Neuronal sensitivity

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The research carried out during the short period of this report represents a continuation of our studies on a previous project supported by the AFOSR (no. 84-0331). This grant (AFOSR 88-0004) was terminated at our request due to transfer of the Principal Investigator to another institution, where this work now continues. In the main study carried out during the period of this report we have found that primary neurons, cultured from the neostriata of embryonic mouse brain, store ATP within synaptic vesicle and release it by exocytosis upon stimulation. It was also found that those cells posses ecto-protein kinase, and that specific phosphoprotein substrates for this activity are present at the cell surface. These findings open for investigation the role of extracellular protein phosphorylation in the regulation and adaptation of neurons in the Central Nervous System.

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FINAL SCIENTIFIC REPORT

Principal Investigator: Yigal H. Ehrlich, Ph.D.

Period of this report: November 1, 1987 to June 30, 1988.

1). concise summary

The main project carried out in our laboratory during the period covered by this report has been focussed on the finding that neural cells posses an ecto-protein kinase activity, which phosphorylates proteins localized at the outer surface of the plasma membrane. We have discovered this activity while carrying a previous project supported by the Air Force Office of Scientific Reserch (AFOSR 84-0331). The report submitted here summerizes the continuation of this resarch during the period Nov.1987-June 1988. The main new findings reported here are that primary CNS neurons, cultured from the neostriatum of embryonic mouse brain, have an ecto-protein kinase and surface phosphoprotein substrates for its activity. These cells were also found to store ATP within synaptic vesicles and secrete it in a calcium-dependent manner upon stimulation. These results open for investigation the role of extracelluar protein phosphorylation in the regulation and adaptaion of CNS neurons.

Two additional projects were pursued in our laboratory in parallel to the studies summarized above. We have continued our investigation on the role of extracellular protein phosphorylation in the regulation of the uptake of norepinephrine (NE). In these studies we found that neural cells of the clone PC12 have ecto-protein kinase activity, and that addition of ATP-gamma-S to the medium significantly stimulates high affinity NE-uptake by these cells. During the same period we have completed the first phase of our investigation on the interaction of the alkyl-ether phospholipid platelet-activating-factor (PAF) with neuronal cells. We have reported that PAF stimulates Ca^{++}-uptake in the neural cells of the lines NG108-15 and PC12 . This effect appears to be mediated by the activity of receptor-operated calcium channels. Since in previous studies we found in NG108-15 and N1E-115 similar stimulation of Ca^{++}-uptake by extracellular ATP, future studies should investigate the interactions between these two systems.

2) Research Objectives.

The research proposal which led to award no. AFOSR 88-004 included studies designed for a three (3) years period. Six specific aims were listed in the original application for this 3 years period, as follows:

1. Biochemical characterization (Kinetic properties, ionic requirements, regulating factors) of the extracellular protein phosphorylation systems operating in primary CNS neurons grown and differentiated in-culture.

2. Determination of the effects of cell stimulation by neurotransmitters, depolarizing agents, neurohormones and growth factors on the activity of the phosphorylation systems defined in specific aim no. 1.

3. Isolation and purification to apparent homogeneity of specific components (ecto-protein kinase, certain protein substrates and phosphoprotein phosphatase) of the neuronal extracellular protein-phosphorylation systems.

4. Development of poly- and mono-clonal antibodies to specific components isolated under specific aim no. 3.

5. Identification of the extracellular protein phosphorylation systems associated with the function of mature synapses, by determining the developmental pattern of these systems during synaptoenesis in-culture (1-18 DIV).

6. Investigation on the role of the phosphorylation of N-CAM by ecto-protein kinases in neuronal function.

(3) Research Accomplishments.

During the period covered by this report we have made progress in one major project of our research and in two ancillary projects related to this research. The major project was studies of ATP secretion and extracellular protein phosphorylation by CNS neurons in primary culture. The two ancillary projects were: (a) Regulation of norepinephrine uptake by extracellular ATP, and (b) Regulation of calcium uptake in cultured neural cells. The main results obtained in these studies are summarized below.

The significant role of secreted ATP in the regulation of neuronal function and the activity of ecto-protein kinases which utilize extracellular ATP to phosphorylate proteins localized at the cell surface have been previously studied in peripheral neurons and in cloned neural cell lines. During the research period reported here we have utilized neostriatal neurons differentiated in primary culture to demonstrate vesicular secretion of ATP and phosphorylation of proteins by extracellular ATP in neurons derived from the central nervous system (CNS). Neostriatal neurons from embryonic mice were maintained in a chemically defined medium for 15-18 days. Functional differentiation was determined by measuring evoked GABA-release. ATP-secretion was measured by luciferin-luciferase assays, and protein phosphorylation by adding γ-^{32}P-ATP to the

extracellular medium. Depolarization by 50mM KCl induced a Ca^{++}-dependent ATP release, and stimulation by 100μM veratridine resulted in secretion of ATP that could be blocked by tetrodotoxin. Phosphorylation of specific protein components with apparent molecular weights of 110K, 80K, 55K, and 30K was detected in striatal neurons incubated for 15 mins with γ-^{32}P-ATP added to the medium, but not by labeling intracellular ATP pools with equivalent amounts of radiactivity presented as inorganic ^{32}Pi. These results open for investigation the role of extracellular protein phosphorylation systems in processes underlying the responsiveness of CNS neurons to secreted ATP. A full account of this study has been published, and 6 copies of the paper are enclosed with this report.

In another study carried out during this period, the potential modulation of norepinephrine (NE) uptake by extracellular ATP and divalent cations was examined in PC12 cells. An analog of ATP, ATPγS, was chosen for these studies since ATPγS can be utilized by protein kinases and the resulting thiophosphate bonds are resistant to phosphatase activity. Previous work in our laboratory has shown that both ATP and ATPγS (0.1 μM) can stimulate NE uptake. In this study, cells were incubated for 5 min at 37^{0}C, in a Krebs-Ringer buffer conatining 1.87 mM Ca^{2+} and no added Mg^{2+}, with and without 1 μM ATPγS. The cells were then washed and NE uptake assayed 30 min later. ATPγS produced a persistent stimulation of uptake and this effect was dependent on the presence of Ca^{2+} in the pretreatment medium. Preincubation with ATP produced no increase in uptake, suggesting that there was an inactivation by dephosphorylation. Pretreatment with ATPγS and an excess of ATP resulted in the inhibition of the ATPγS-induced stimulation. The ATPγS pretreatment protocol was used to examine kinetic changes and the results showed an increase in the Vmax, with no change in the apparent Km for NE. Experiments are currently in progress to determine ecto-protein kinase activity in PC12 cells, to identify the surface proteins phosphorylated by ATPγ^{35}S, and examine their role in action of the NE-transporter system and its regulation by extracellular ATP.

We have reported previously that extracellular ATP can stimulate calcium uptake into neural cells and cause an increase in the levels of intracellular free Ca^{++}-ions. It was suggested that the mechanism involves regulation of receptor-operated calcium channel. A potent regulator of such mechanism in divere biological systmes is the naturally occuring alkyl-ether phospholipid called platelet activating factor (PAF). However, whether this critical extracellular mediator plays a role in neuronal function has not been known. In the present study we have investigated the interactions of PAF with cloned neural cells. We have found that PAF increased the intracellular levels of free calcium ions in cells of the clones NG108-15 and PC12. The increase was dependent on extracellular calcium and was inhibited by the antagonistic PAF analog CV-3988 and by the calcium-influx blockers prenylamine and diltiazem. A functional consequence of this interaction was revealed by measuring a PAF-elicited, Ca^{++}-dependent secretion of adenosine triphosphate from PC12 cells. Exposure of NG108-15 cells for 3 to 4 days to low concentrations of PAF induced neuronal differentiation; higher concentrations were neurotoxic. Thus, by influencing Ca^{++} fluxes, PAF may play a physiological role in neuronal development and a pathophysiological role in the degeneration that occurs when neurons are exposed to circulatory factors as a result of trauma, stroke, or spinal cord injury. These effects of PAF appear to be mediated by the activity of receptor-operated calcium channels. Continued

Studies examine the role of extracellular ATP secreted by neurons in the regulation of this receptor-coupled activity in neurons.

(4) Written Publications.

Jin Zhang, Elizabeth Kornecki, Joany Jackman and Yigal H. Ehrlich. "ATP Secretion and Extracellular Protein Phosphorylation by CNS Neurons in Primary Culture." Brain Research Bulletin, Vol. 21, pp. 459-464. (1988).

J.E. Chaffee, Y.H. Ehrlich, E.D. Hendley. "Stimulation of Norepinephrine uptake by ATP in PC12 cells." Society for Neuroscience Abstracts. Vol 14, no 275.10 (1988).

Y.H. Ehrlich, I. Galbraith, J. Jackman and E. Kornecki. "Ecto-protein kinase at the surface of CNS neurons." Society for Neuroscience Abstracts Vol. 14, no 372.8 (1988).

Brain Research Bulletin, Vol. 21, pp. 459–464. © Pergamon Press plc, 1988. Printed in the U.S.A. 0361-9230/88 $3.00 + .00

ATP Secretion and Extracellular Protein Phosphorylation by CNS Neurons in Primary Culture

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ZHANG, J., E. KORNECKI, J. JACKMAN AND Y. H. EHRLICH. *ATP secretion and extracellular protein phosphorylation by CNS neurons in primary culture.* BRAIN RES BULL 21(3) 459–464, 1988.—The significant role of secreted ATP in the regulation of neuronal function and the activity of ecto-protein kinases which utilize extracellular ATP to phosphorylate proteins localized at the cell surface have been previously studied in peripheral neurons and in cloned neural cell lines. In the present study we have utilized neostriatal neurons differentiated in primary culture to demonstrate vesicular secretion of ATP and phosphorylation of proteins by extracellular ATP in neurons derived from the central nervous system (CNS). Neostriatal neurons from embryonic mice were maintained in a chemically defined medium for 15–18 days. Functional differentiation was determined by measuring evoked GABA-release. ATP-secretion was measured by luciferin-luciferase assays, and protein phosphorylation by adding γ-^{32}P-ATP to the extracellular medium. Depolarization by 50 mM KCl induced a Ca^{++}-dependent ATP release, and stimulation by 100 μM veratridine resulted in secretion of ATP that could be blocked by tetrodotoxin. Phosphorylation of specific protein components with apparent molecular mass of 110 Kd, 80 Kd, 55 Kd, 30 Kd and 20 Kd was detected in striatal neurons incubated for 15 min with γ-^{32}P-ATP added to the medium, but not by labeling intracellular ATP pools with equivalent amounts of radioactivity presented as inorganic ^{32}Pi. These results open for investigation the role of extracellular protein phosphorylation systems in processes underlying the responsiveness of CNS neurons to secreted ATP.

Striatal neurons Primary neuronal cultures [^{3}H]GABA release ATP secretion Ecto-protein kinase Protein phosphorylation

A large body of evidence documents the significant role of protein phosphorylation systems in the regulation and adaptation of multiple neuronal functions (6, 8, 11, 17, 18, 22). In this process the enzyme protein kinase transfers the gamma phosphate of ATP to specific sites in various intracellular proteins (21,24). In addition, conditions necessary for phosphorylation of *extra*cellular proteins also exist in the nervous system (11). It is well known that ATP is stored within synaptic vesicles of adrenergic, cholinergic and purinergic neurons, and secreted to the synaptic cleft by exocytosis when the neurons are stimulated (2, 4, 16, 23, 25, 28, 30, 31, 33). Recently, we have presented evidence that neural cells have an ecto-protein kinase which utilizes extracellular ATP to phosphorylate specific proteins localized at the cell surface (9,10). These studies were carried out with cloned neural cells of peripheral origin. Investigation of the role of extracellular protein phosphorylation systems in the regulation of brain function must be initiated by demonstrating this activity in neurons derived from the central nervous system (CNS). In the present report we demonstrate that embryonic neostriatal neurons grown in primary culture under conditions that induce neuronal differentiation and synaptogenesis (29), secrete ATP upon depolarization and can utilize extracellular ATP to phosphorylate specific protein components. These findings provide the data-basis necessary for investigating the role of extracellular protein phosphorylation activity in neuronal development, and in the feedback regulation of synaptic functions by ATP secreted from differentiated CNS neurons. A preliminary report of this study has been presented (32).

METHOD

Primary Neuronal Cultures

Neostriata were dissected from the brain of 14-day mouse embryos, mechanically dissociated, plated in 6-well cluster plates ($0.8–1.0\times10^6$ cells/ml; 2 ml/well), and maintained in a

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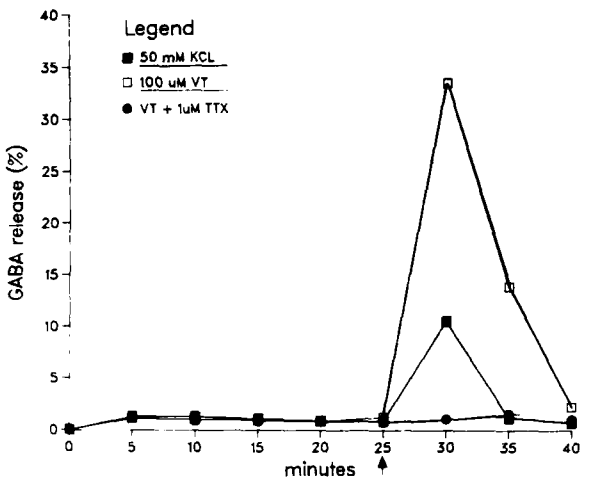

FIG. 1. Depolarization-induced release of [^{3}H]GABA from differentiated neostriatal neurons in primary culture. Striata dissected from 14-day mice embryos were dissociated and the cultured neurons maintained in a chemically defined medium for 14–15 DIV. Attached cells were preloaded with [^{3}H]GABA in KRB for 10 min at 37°C (see the method section). After two washes at time-point zero the cells were covered with KRB, aliquots of the medium were collected every 5 min and subjected to scintillation counting. Arrow indicates the time point at which KRB containing 50 mM KCl (in place of NaCl), or KRB containing 100 μM veratridine (VT) without or with 1 μM tetrodotoxin (TTX) was added to the wells (TTX was added 5 min before the VT). GABA release is expressed in % of total [^{3}H]GABA uptake by the cells in each well (15). Data shown are the means of 4–12 experiments for each incubation condition, in which every point was assayed in triplicate. The SEMs were less than 10% of the mean (see also Table 1).

chemically defined, serum-free medium for 14–21 days, as described by Weiss *et al.* (29). Under these conditions, cultures maintained for 14 days in vitro (DIV) were found to contain over 90–95% neurons and revealed a high level of neuronal differentiation, the presence of presynaptic vesicles and defined postsynaptic elements (29). In the experiments reported here, these neurons were studies at 15–18 DIV.

Measurement of GABA release was conducted at 37° essentially as described by Gallo *et al.* (15). In brief, neostriatal cells attached to the plate were loaded with 0.5 μCi/well of [^{3}H]GABA (NEN) in Krebs-Ringer buffer (KRB) during a 10 min preincubation period as described in (15). After 2 gentle washes with KRB, basal release was monitored over a 25 min period at 5 min intervals. Evoked release was initiated by replacing the buffer with KRB supplemented with 50 mM KCl (in place of NaCl), or with KRB containing 100 μm Veratridine. A 5 min releasate was collected, followed by 2×5 min incubation in KRB. [^{3}H]GABA in aliquots of the collected extracellular medium and in washed cells extracted with NaOH (15) was determined by liquid scintillation counting. Release data are expressed as a percentage of the total [^{3}H]GABA taken up by the cells in each well (15).

ATP release was measured using the protocol described above for GABA release, except that aliquots (0.45 ml) of the medium collected at each time point were immediately chilled on ice and then subjected to ATP determination by adding 50 μl of a commerical luciferin-luciferase reagent (Chrono-Lume; Chronolog, Havertown, PA) containing synthetic luciferin, and luciferase prepared from firefly tails. Luminescence produced by ATP in the luciferin-luciferase reaction was detected by a photomultiplier tube of a lumiaggregometer (Chronolog, PA). A standard curve of ATP measured under the same ionic conditions was used to quantitate ATP concentration in the releasate, expressed in nM ATP.

Protein phosphorylation reactions were performed at 37°C with γ-^{32}P-ATP added to the extracellular medium, using attached neostriatal cells maintained in serum-free medium for 15–18 DIV. The procedures described by Ehrlich *et al.* (9) for ectokinase assays with NG108-15 cells were followed, except using 12-well cluster plates instead of 96-well plates and a final reaction volume of 600 μl. Cells were rinsed twice with KRB and then covered with buffer containing 10 μCi of γ-^{32}P-ATP (ICN) and a final ATP concentration of 1 μM. Following incubation for 15 min at 37°C, the reaction was terminated by adding "SDS-stop solution" (7) and the proteins separated by slab-gel electrophoresis using 7–14% exponential polyacrylamide gel gradients, as described previously (9). Autoradiograms were used to quantitate ^{32}P-incorporation into specific protein bands by scanning with a digitizing laser microdensitometer (LKB Ultroscan XL). Molecular weights (MW) of phosphoproteins were estimated by determining relative mobility compared to protein standards (BioRad) separated on the same gel and detected by staining with Coomassie-blue (7–9).

RESULTS

Striatal neurons were maintained in primary culture in a chemically defined medium for 15–18 days in vitro, as detailed in the Method section. Previous studies using these culturing conditions have demonstrated morphological differentiation and synaptogenesis in these cells during 10–14 DIV (29). To demonstrate a functional aspect of neuronal maturation in these cultures, we measured depolarization-induced neurotransmitter release in attached cells preloaded with [^{3}H]GABA. Figure 1 demonstrates that under non-stimulating conditions, a stable basal release is exhibited by the cells, which can be maintained for at least 40 min. When the cells were stimulated with 50 mM KCl, about 10-fold increase in [^{3}H]GABA release over basal conditions was evident. An even more pronounced release of GABA was evoked by stimulating the cells with 100 μM veratridine, and the veratridine-induced GABA release was completely blocked by 1 μM tetrodotoxin (TTX) (see Fig. 1).

The experimental protocol used to measure GABA release as shown in Fig. 1 was followed for measuring ATP-release by a luciferin-luciferase assay carried out with aliquots of medium collected at 5 min intervals from attached neostriatal neurons incubated in Krebs-Ringer buffer. Under nonstimulating conditions, ATP concentration in the medium was found to be about 0.5 nM (see bar marked C in Fig. 2). In the releasate collected during 5 min incubation after replacement of the medium with KRB containing 50 mM KCl (in place of NaCl), the ATP concentration was 17.3±4.3 nM. Figure 2 also shows that veratridine induced about a 50-fold increase in ATP rlease (23.7±1.1 nM ATP), and over 80% of the veratridine-evoked ATP release was blocked by TTX. The data presented in Table 1 demonstrate that 47% of the [^{3}H] GABA released by cells depolarized with 50 mM KCl can occur in a Ca^{++}-free KRB medium,

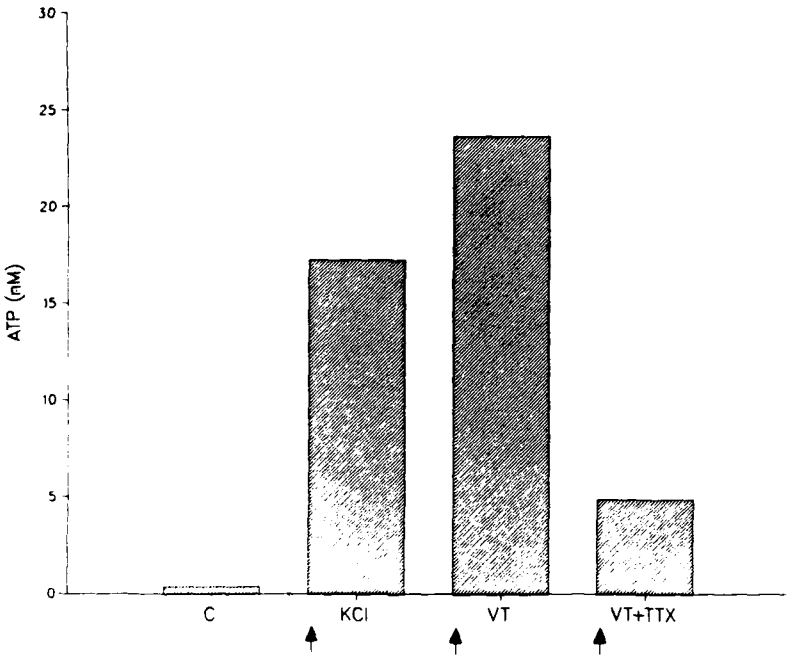

FIG. 2. Secretion of ATP from striatal neurons in primary culture. Cells at 14–15 DIV were assayed following the protocol shown in Fig. 1 and detailed in its legend. ATP concentration in aliquots of the collected medium was determined by luciferin-luciferase reactions as described in the Method section. Bars show the mean data obtained under basal (C) and stimulated conditions. Arrows indicate the time point of stimulation by 50 mM KCl, 100 μM veratridine (VT) and VT+TTX (1 μM), corresponding to the arrow depicted in Fig. 1. Data shown are means from 4 experiments; SEMs are provided in the text and Table 1.

TABLE 1

DEPOLARIZATION-INDUCED SECRETION OF ATP AND GABA FROM STRIATAL NEURONS: DEPENDENCE ON Ca^{++} IN THE EXTRACELLULAR MEDIUM

Incubation-Conditions*	Incubation in KRB	Incubation in Ca^{++}-free KRB	Ca^{++} dependence
ATP-Secretion (in nM; mean ± SEM)	17.3 ± 4.3 (n=4)	4.4 ± 1.5 (n=3)	75%
[^{3}H]GABA-Release (in % of total uptake)	10.52 ± 0.86 (n=12)	4.95 ± 0.15 (n=4)	53%

*Neostriatal cells at 14–15 DIV were stimulated with 50 mM KCl in normal KRB or in KRB prepared without $CaCl_2$, and the medium collected after 5 min incubation at 37°C as described in the legend to Fig. 1. ATP and [^{3}H]GABA release were measured as detailed in the Method section. n = the number of experiments.

whereas 75% of the depolarization-induced ATP release is Ca^{++}-dependent.

Since striatal neurons secrete ATP, the cosubstrate for protein kinase activity, they may also possess extracellular protein phosphorylation systems. To measure the phosphorylation of proteins by extracellular ATP we added γ-^{32}P-ATP to the medium covering intact striatal neurons while still attached to their substratum. In control wells of the same cluster plate, the intracellular ATP pools of the cells were labeled by adding an equivalent amount (in μCi) of radioactive, inorganic ^{32}Pi and incubating for the same time period (15 min). Figure 3 depicts an autoradiogram of ^{32}P-containing protein bands labeled by these two procedures. It can be seen that under these incubation conditions proteins

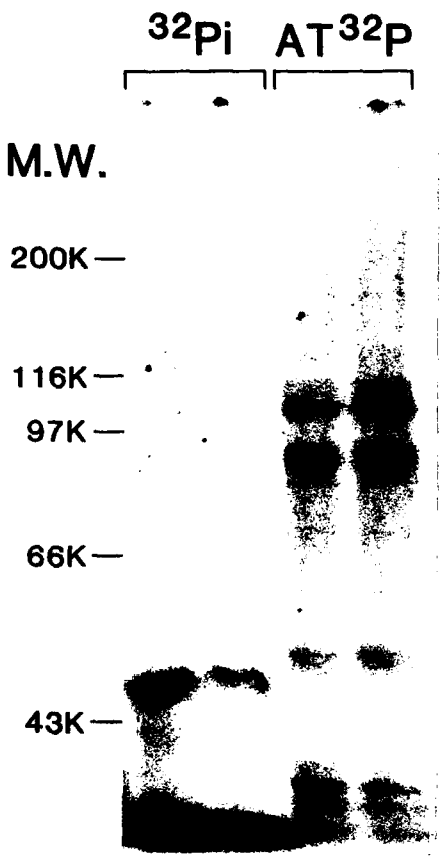

FIG. 3. Autoradiogram of phosphorylated proteins in neostriatal cells incubated for 15 min at 37°C with 60 μCi per well of γ-^{32}P-ATP (ATP) or inorganic ^{32}Pi (Pi) in KRB. Reactions were carried out as described in the Method section and terminated by adding SDS-stop solution. Solubilized, reduced proteins [2% beta-mercaptoethanol; 5 min/95°C, (7)] were resolved in 7–14% exponential gradient of polyacrylamide. The gel was autoradiographed with Kodak X-Omat film for 6 days. Each sample is shown in duplicate. Representative of 4 experiments with neostriatal cells assayed at 15–18 DIV. Mobility of standard MW markers is shown on the left.

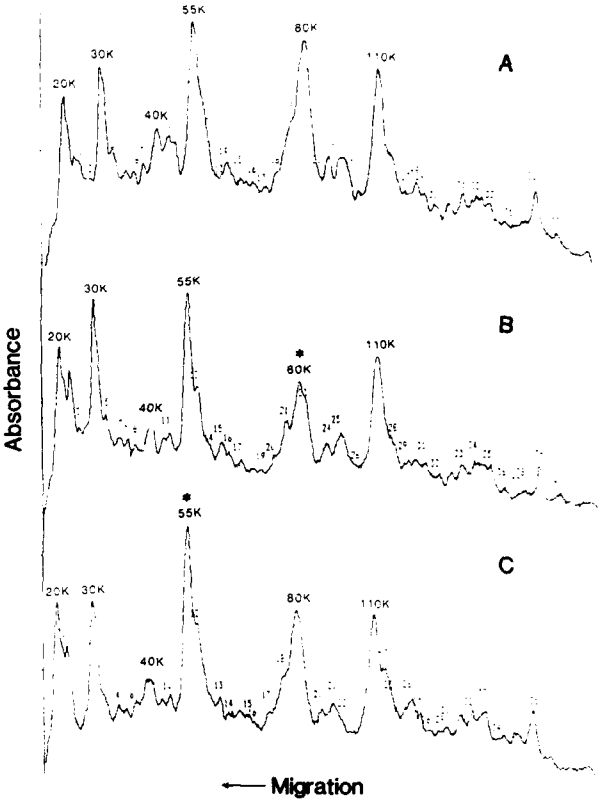

FIG. 4. Phosphorylation of proteins in neostriatal neurons by extracellular γ-^{32}P-ATP added in normal KRB (A), in KRB containing 50 mM KCl (B), or 100 μM veratridine (C). Presented are densitometric scans (using LKB-Ultroscan) of autoradiograms obtained as described in Fig. 3, except adding 10 μCi/well and exposing slab gels to X-ray film for 12–13 days.

with apparent molecular mass of 110,000 kilodaltons (Kd), 80 Kd, 55 Kd and approximately 30 Kd and 20 Kd incorporated ^{32}P from γ-^{32}P-ATP added to the extracellular medium, but *not* by labeling the neurons for the same period with equivalent amount of radioactivity provided as ^{32}Pi. Densitometric scans of the autoradiograms of separated proteins from neostriatal cells incubated for 15 min with γ-^{32}P-ATP added in normal KRB, in high K^+-KRB, and in KRB supplemented with 100 μM veratridine are shown in Fig. 4A, 4B and 4C, respectively. The results of these experiments (n=8) were quantitated by measuring peak areas (see Table 2). This analysis revealed that compared to basal incubation conditions, depolarization of the cells by 50 mM K^+ caused a significant decrease in the phosphorylation of a protein component with apparent MW of 80 K. In contrast, ^{32}P-*incorporation from extracellular* γ-^{32}P-*ATP into a 55 K* protein increased significantly in striatal neurons stimulated with veratridine. Functional studies of ecto-protein kinase activity in differentiated CNS neurons can now focus on these specific phosphoprotein components.

It is important to point out that in striatal neurons that were assayed at 15–18 DIV (namely, *after* differentiation and synaptogenesis), only minimal phosphorylation by extracellular ATP was detected in proteins with molecular mass greater than 110 Kd (see Fig. 4), which comigrate with neuronal cell adhesion molecules (N-CAMs). On the other hand, pronounced phosphorylation of N-CAMS by ecto-protein kinase activity was found during the phase of rapid neurite extension in cloned neural cells differentiating in culture (9). Similar experiments, to be reported in detail elsewhere, have found phosphorylation by extracellular ATP of proteins recognized by anti D_2-CAM antibodies (9) in CNS neurons maintained in primary culture for 4–6 DIV, namely, *during* neuronal differentiation. These results suggest that in the nervous system phosphorylation of different proteins by ecto-kinase activity is developmentally regulated.

DISCUSSION

Procedures for obtaining pure neuronal populations by culturing cells from central nervous tissue in chemically defined, serum-free media have enabled detailed investigation of various molecular aspects of the development, differ-

TABLE 2

EFFECTS OF HIGH K^+ AND VERATRIDINE STIMULATION ON PROTEIN PHOSPHORYLATION BY EXTRACELLULAR ATP

Incubation Conditions	^{32}P-Incorporation Into Specific Protein Components						Significant Change in %
	20 K	30 K	40 K	55 K	80 K	110 K	
Basal (KRB) (n=8)	4.99* ±0.51	5.95 ±0.44	4.29 ±0.57	6.33 ±0.24	7.53 ±0.50	7.98 ±0.50	
50 mM KCl (n=8)	5.48 ±0.25	6.83 ±0.14	4.03 ±0.36	6.41 ±0.35	5.33† ±0.62	8.34 ±0.54	−29% $p<0.01$
Veratridine (n=8)	5.96 ±0.43	6.90 ±0.22	3.66 ±0.32	7.64† ±0.30	7.39 ±0.30	8.33 ±0.43	+21% $p<0.005$

*Values of ^{32}P-incorporation are means ± SEM for the number of experiments shown (n), expressed as percentage of relative area of specific peaks obtained by digitizing desitometric analysis of autoradiograms, as described in the Method section.

†Denotes statistically significant difference from basal incubation conditions, calculated by Student's *t*-test. Neostriatal neurons were grown and assayed as detailed in the legend to Fig. 4.

entiation and function of intact CNS neurons (1, 14, 15, 27). Weiss *et al.* (29) has employed defined conditions for culturing striatal neurons from embryonic mouse brain, and using immunocytochemical and electron-microscopic criteria demonstrated morphological differentiation and synaptogenesis in these cultured neurons during 10–14 days in vitro. In the present study, we have used these procedures for culturing striatal neurons, and demonstrated their functional differentiation by measuring depolarization-induced neurotransmitter release and veratridine-evoked, TTX-blocked secretion of GABA and ATP from cultured striatal neurons at 15–18 DIV.

Evoked secretion of ATP stored in synaptic vesicles of cholinergic, adrenergic and purinergic neurons has been previously shown in the peripheral nervous system (PNS) (2, 4, 16, 25, 28, 31, 33) and in synaptosomes isolated from the CNS (23,30), but not in intact CNS neurons differentiated in-culture. In the present study we found that the release of ATP from cultured striatal cells meets several criteria of evoked vesicular secretion from mature neurons. The finding that stimulation of striatal neurons causes secretion of ATP indicates that in the CNS, as in the PNS, extracellular ATP may exert feedback control over the activity of the secreting nerve terminals (26), as well as providing a transient signal that can be transmitted to postsynaptic target cells (20). It should be emphasized that the absolute concentrations of released ATP reported here (Fig. 2 and Table 1) were determined in a relatively large volume used to cover a monolayer of cultured cells, and without protecting released ATP from hydrolysis. It may be expected, therefore, that the actual value of extracellular ATP concentration produced in the synaptic cleft during neuronal stimulation is substantially higher than the nM values reported in Fig. 2 and Table 1.

ATP secreted by neurons can be hydrolyzed by ectonucleotidases to adenosine which interacts with P_1-receptors, or act by binding to P_2-purinoreceptors that respond directly to ATP and to unhydrolyzable ATP analogs (2,3). In addition, neuromodulatory effects of extracellular ATP which require native, hydrolyzable ATP were demonstrated both presynaptically (26) and postsynaptically (20). Such modulation may be mediated by extracellular protein phosphorylation systems in which a membrane-bound ecto-kinase and/or a soluble, released exo-kinase utilize secreted ATP to phosphorylated cell surface proteins or components of the extracellular matrix (11,12). Previous studies have demonstrated extracellular protein phosphorylation in cloned neural cells, NG108-15 and PC12 (5, 9–11). In the present study we demonstrated that differentiated CNS neurons cultured from embryonic mouse neostriatum, which secreted ATP on stimulation, can utilize extracellular ATP in the phosphorylation of specific protein components. These proteins were not labeled when the cells were incubated for the same period with equivalent amounts of ^{32}Pi, used to label intracellular ATP pools (Fig. 3). These results indicate that the protein phosphorylation seen in striatal neurons incubated up to 15 min with extracellular γ-^{32}P-ATP cannot be attributed to ^{32}Pi liberated by ecto-ATPase, and that the phosphorylation sites in the proteins labeled under these conditions are located extracellularly. The finding that stimulation by a depolarizing KCl concentration and by veratridine (both of which induce ATP release in these cultures, see Fig. 2) result in opposing effects on the phosphorylation of specific protein components by extracellular AT^{32}P (Fig. 4), suggests that these effects are not simply a consequence of the release of ATP, but may be involved in the mechanisms underlying two different biochemical pathways of stimulus-secretion coupling.

Previous studies of peripheral neuronal systems (20,26), cloned neural cell lines (5, 9, 10) and synaptosomes (19) have implicated extracellular ATP and ecto-protein kinase activity in neuronal development [phosphorylation of N-CAMs, (9)], in the regulation of neurotransmitter release (26) and reuptake (19), in stimulating Ca^{++}-influx (10) and in the modulation of neuronal signal transduction systems involved in synaptic plasticity (13). The present results open for investigation the role of secreted ATP and of extracellular protein phosphorylation systems in the regulation and adaptation of these neuronal functions in the central nervous system.

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